Title: Intussusceptive pillar formation in developing porcine glomeruli

Authors: Anastasia Logothetidou[1], Ward De Spiegelaere[1], Tim Vandecasteele[1], Waltraud Tschulenk[2], Ingrid Walter[2], Wim Van den Broeck[1], Pieter Cornillie[1*]

Address of the authors:
[1] Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[2] Institute of Anatomy, Histology and Embryology, Department of Pathobiology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

Running title: Intussesceptive pillars in porcine fetal glomeruli

***Corresponding author**
e-mail: pieter.cornillie@ugent.be, tel. +32 9 264 77 11, fax: +32 9 264 77 90
Address: Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Abstract

Background/Aims: Intussusceptive angiogenesis (IA) is a dynamic process which contributes to vascular expansion and remodeling. Intraluminal pillars have long been the distinctive structural indicator of intussusceptive angiogenesis. However, the mechanism of their formation has not been fully elucidated yet.

Methods: Using light and electron microscopy, we studied intussusceptive vascular growth in the developing porcine metanephric kidney.

Results: We observed intraluminal pillars formed by endothelial cells in the vasculature of developing glomeruli. Their diameter was < 2.5 µm, consistent with the diameter of nascent pillars. TEM revealed that the majority of these pillars consisted only of endothelium. However, a central core of extracellular matrix (ECM) covered by endothelium, reminiscent of a more mature intussusceptive pillar, was also found in the lumen of a glomerular capillary. Perivascular cells or pericytes were not involved in the pillar structure during these stages of formation.

Conclusion: This study shows ECM presence in a mature intussusceptive pillar without any perivascular cell involvement in the structure. This leads to the hypothesis that ECM deposition preceeds the participation of these cells in the formation of intraluminal pillars during IA in porcine metanephric glomerular capillaries.

Key words: Intussusceptive angiogenesis, intraluminal pillar, metanephros, glomerulus

Introduction

Angiogenesis, the process through which the vascular system expands, occurs mostly during development and cyclic organ growth but also in pathological conditions involving tissue repair, organ regeneration, chronic inflammation and tumorigenesis [1-11]. The two best known angiogenic mechanisms, i.e. sprouting and intussusceptive angiogenesis (IA), lead to the expansion of the capillary network. However, they involve different cell types and are regulated by different molecules [4]. Sprouting angiogenesis is responsible purely for vascular growth; yet, IA can also involve vascular remodeling through pruning of excessive blood vessels [12]. IA was first observed in the pulmonary capillaries of neonatal rats by scanning electron microscopy when numerous holes of 1–2 μm in diameter were detected within vascular corrosion casts [13]. These holes corresponded to thin transcapillary (intraluminal) tissue pillars which lead to the division of a single blood vessel into two new vessels [13, 14]. Intraluminal pillars are considered the characteristic features of the morphogenetic process of IA [15-18].

Intussusceptive microvascular growth (IMG) refers to vessel network formation by insertion of tissue columns facilitating rapid expansion and an increase in the complexity of capillary beds. Intussusceptive arborization (IAR) can be recognized by the occurrence of a series of pillars and is involved in the differentiation of parts of the capillary plexus into immediate pre- and postcapillary vessels. Finally, intussusceptive branching remodeling (IBR) involves remodeling via an expansion or pruning of vessel branches and an optimization of the branching geometry and the hemodynamic conditions of the vascular tree [19].

Despite their diversity, all types of IA are characterized by the formation of intraluminal pillars which occurs in four phases [14]. In the first phase, a zone of contact between opposite capillary walls is created (formation of a transcapillary interendothelial bridge). In the second phase, the intercellular junctions of the endothelium are reorganized and the endothelial bilayer is centrally perforated. In the third phase, an interstitial pillar core is formed and successively invaded by cytoplasmatic extensions of myofibroblasts and pericytes and subsequently by interstitial fibers. In the last phase, the slender pillar grows and fuses with adjacent pillars (pillar diameter > 2.5 μm). The process of IA eventually leads to remodeling and separation of the initial capillary into two capillaries.

In pathological tissues such as tumors, various mechanisms of angiogenesis have been identified [20]. In murine ascites tumor vessels, intraluminal bridging has been described during which endothelial cytoplasmic processes extend into and across the vessel lumen, forming transluminal bridges that divide blood flow into multiple smaller-sized channels [21]. More specifically, in the vasculature of experimental subcutaneous tumors, a detailed model of vascular division due to endothelial bridging was proposed by Paku et al. [22]. During this mechanism, endothelial bridges are formed and subsequently the bridge-forming endothelium attaches to a type I collagen bundle in the underlying connective tissue. The actin cytoskeleton of the endothelial cell then exerts a pulling force to the collagen bundle, resulting in the transport of the latter through the vessel lumen. This process, named inverse sprouting, generates a connection between the processes of endothelial bridging and intussusceptive angiogenesis and identifies the collagen-pulling force behind pillar formation.

In the present study, the presence of IA and the pillar ultrastructure were investigated in the glomerular capillaries of the porcine metanephric kidney at different developmental stages using serial sectioning combined with light and electron microscopy. Previous studies have shown that IA is active during renal and glomerular development in vertebrates [18, 23]. The numerous glomeruli in the kidney are easily identifiable and delineable regions in the cortex which allows easier sampling. Moreover, the developing kidney enables the investigations of different stages of vascular development, as the formation of nephrons proceeds in a centrifugal pattern so that the newly formed nephrons are found in the superficial cortex whereas the oldest and most mature nephrons are located in the juxtamedullary area regardless of the fetal age [24, 25].

Methods

Sample collection and processing

Porcine fetuses of different developmental stages were obtained from gravid uteri collected in a local slaughterhouse and their approximate age in embryonic days postconception (E) was calculated from their crown-rump length (CRL) [26]. The fetuses used in this research had a CRL of 6.5 cm (E46), 9.5 cm (E55), 14 cm (E64) and 22 cm (E98). For both light and electron microscopic studies, small pieces ± 1 mm^3 of fetal kidney cortex were fixated overnight in Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0,1 M sodium cacodylate buffer, pH 7.2). The tissue blocks were then postfixated in 1% reduced osmium tetroxide for 1.5 h, dehydrated through ascending concentrations of ethanol and embedded in EPON 812 resin.

Light microscopy and 3D reconstruction of semithin sections

One kidney sample of each of the above mentioned embryonic ages (n = 4) was used for serial semithin sectioning which was performed as described by Ruthensteiner [27]. Briefly, after the application of glue, i.e. Pattex gel compact diluted with a few drops of xylene, on one side of the EPON block, a diamond knife with a big boat (DiATOME, histo jumbo, ultra 45° 8 mm) was used to cut serial semithin sections of 0.5 µm. As the sectioning progressed, the sections formed a ribbon due to the glue in between and were later detached from the knife and transferred onto pretreated glass slides. After stretching and drying, the ribbons were stained with toluidine blue, mounted with DPX and observed with an Olympus BX61 (Olympus, Belgium) microscope. All the glomeruli present in each serial section were captured by an Olympus BX-UCB camera, followed throughout > 200 semithin sections and analyzed for the presence of pillars. Endothelial protrusions in the capillary lumen were considered as intraluminal pillars only when they appeared and then disappeared in consecutive sections, spanning from one endothelial wall towards the opposite one. In that way, other structures such as vessel bifurcations, endothelial folds or artifacts were not accidentally attributed as pillars. Digitized images of areas of interest were transferred to the Amira 6.1.1 software program (FEI, France) using the protocol described by Cornillie et al. [28] in order to perform three-dimensional (3D) reconstruction of the pillar formation.

Transmission electron microscopy (TEM) and 3D reconstruction of ultrathin sections

Two additional fetal kidney samples of E64 and E98 (n=2) were used for ultrathin sectioning starting at a random point in the tissue block. An ultra 45° 2.5 mm diamond knife (DiATOME, Switzerland) was utilized to cut 150 consecutive ultrathin sections of ± 80 nm thickness which were then collected on precoated formvar copper grids. Sections were contrasted with 1% uranyl acetate in 10% ethanol and afterwards with a lead citrate buffer (133 mg lead nitrate and 175 mg sodium citrate in 10 ml double distilled water). The sections were viewed at 80 kV with a JEM-1400 Plus transmission electron microscope (Jeol, Belgium) equipped with a Quemesa TEM CCD camera (Olympus, Belgium). Image analysis was performed with the Radius software (EMSIS, Belgium). All reagents and materials were purchased from Aurion (Aurion ImmunoGold Reagents & Accessoires, the Netherlands).

Results

Intussusceptive pillars in metanephric glomerular capillaries

The semithin sections of the different fetal kidney samples showed that the metanephros consisted of several tubules in the medullar area and S-shaped bodies and glomeruli in the cortical area. Glomeruli of different developmental stages, i.e. stages III, IV and V were easily discerned depending on their diameter, the abundancy of capillary loops and their position in the kidney cortex (Fig. 1A). The glomerular tuft, the Bowman's capsule and the vascular and urinary poles of the renal corpuscle were clearly identifiable (Fig. 1B). In the 18 glomeruli that were analyzed in total, numerous endothelial protrusions were found in the glomerular capillaries. We were able to prove that 6 of them were indeed pillars, 2 of which were found in stage IV glomeruli and the rest 4 were found in stage V glomeruli. Analysis of the serial sections demonstrated endothelial cytoplasmic processes which elongated in the lumen towards the opposite side of the vessel wall and finally disappeared. Their diameter was always < 2.5 μm which is consistent with the diameter of a nascent pillar. By following serial sections, the point at which a pillar started and its intraluminal extent until the end point could be clearly identified (1C-F). Three-dimensional reconstruction was performed using these serial sections in order to highlight the recreated structures (Fig. 1G).

Ultrastructural changes in glomerular capillaries during development

Ultrastructural changes in the glomerular cells and more specifically in the filtration barrier have been previously described by Friis [29] during postnatal development in porcine. Our TEM analysis of stage III, IV and V glomeruli showed similar findings. The immature capillaries of stage III glomerulusshowed endothelia with a large amount of cytoplasm and abundant euchromatin in their nuclei. The endothelium lacked fenestrations and the podocyte processes were not formed yet (Fig. 2A). In stage IV glomerulus, interdigitations of podocyte processes started to develop and the basal laminae of the endothelial cells and the podocytes were separated further (Fig. 2B). Finally, in stage V glomerulus the mature capillaries exhibited fenestrated thin endothelium with relatively scanty cytoplasmic organelles, a fully-developed glomerular basement membrane and numerous podocyte processes (Fig. 2C).

Ultrastructure of intussusceptive pillars

TEM analysis of 10 glomeruli lead to the identification of 3 nascent pillars which were found in stage V glomeruli. The intraluminal pillars were formed by single endothelial cell processes, whichwere connected to a different part of the vessel wall on the opposite side of the lumen. Interstitial tissue was not present within these nascent pillars (Fig. 3).

Additionally, 1 interesting structure resembling a more mature intraluminal pillar was also identified in the capillary lumen of a stage IV glomerulus. This pillar-like structure consisted of extracellular matrix covered by endothelium and its diameter was ± 1.2 μm (Fig. 4). Moreover, no other types of cells except endothelial cells participated in the formation of both aforementioned pillar structures.

Discussion

The vasculature in the glomerular tuft showed characteristics of intussusceptive angiogenesis, i.e. intraluminal pillar formation, at various stages of development. The regions of interest were followed throughout serial sections to exclude vessel bifurcations or other structures, which may look like intraluminal pillars in single sections. Thus, it was ensured that they represented endothelial pillars with a determined starting and ending point, connecting opposite sides of the endothelial wall.

Although the formation of transluminal pillars is considered the most characteristic feature of intussusceptive angiogenesis [19, 31, 32], the exact mechanism of this process has yet to be completely clarified. In the glomerular capillaries, the pillars originated from an endothelial cell and extended in the lumen towards the opposite side of the vessel wall. This is in contrast with the original hypothesis of intussusceptive angiogenesis, during which opposite endothelial walls form a bridging contact [14]. Furthermore, both maturation stages of pillars demonstrated in the current research showed no involvement of perivascular cells. Therefore, we suggest that, in the very specific vascular setting of the glomerulus, perivascular cells are not a driving force in the initiation of pillar formation mechanism. This is in agreement with the original hypothesis of intussusceptive angiogenesis [14] and the mechanism presented in tumor-induced intussusceptive angiogenesis [22]. Although a fully mature pillar was not identified in our samples, we can hypothesize that podocytes (glomerular perivascular cells) would be involved later in the pillar formation and subsequent vessel splitting. Based on our observations of vascular bifurcations in the glomerular capillaries, the splitting is initiated at the outer side of the capillary, opposite of the side where the mesangial cell resides. Glomerular basement membrane and podocytes follow the invagination of the endothelium, leading to the formation of two capillaries from a pre-existing one (online suppl. Fig. 5; for all online suppl. material, see www.karger.com/doi/10.1159/000490905).

Most pillars identified in this research were nascent pillars, consisting only of endothelium. During pillar maturation, interstitial tissue is involved in the pillar formation. In both physiological and pathological situations, the core of a mature pillar reveals a bundle of collagen fibers [14, 22, 33]. The current study demonstrated a structure resembling a mature pillar and its cross section showed extracellular matrix covered by endothelium. However, the core of this structure did not contain collagen fibers. Since the only type of collagen found in healthy glomeruli is type IV [34, 35], it is most likely that this type is present in the ECM of the pillar. Collagen IV forms meshworks, not fibers, and it is found in the glomerular basement membrane and the mesangial extracellular matrix [36]. Furthermore, endothelial cells, mesangial cells and podocytes of developing glomeruli are known to synthesize different alpha chains of collagen IV [37]. The absence of collagen fibers in the core of the pillar indicates that collagen bundle formation is not necessary for intussusceptive angiogenesis in the metanephric glomerulus which leads to the hypothesis that the mechanism of pillar formation might be tissue-specific.

During glomerulogenesis, capillary loops increase in number and endothelial and podocyte cell layers begin to resemble their fully mature counterparts [30]. Our observations showed similar structural changes in the capillary phenotypes of stage III, IV and V glomeruli. Moreover, of the 18 glomeruli that we used for serial sectioning, we were able to identify 3 pillars in stage IV glomeruli and 7 in stage V glomeruli, in total. In our previous research [23] using vascular corrosion casts of fetal kidneys, we found typical holes in the casts representing transcapillary pillar formation in all 3 developmental stages of glomeruli, although the majority of the holes was seen in mature glomeruli. This led us to believe that the onset of IA occurs in stage III glomerulus but in the current research we were not able to identify any pillars in stage III glomeruli. It is possible that there is a correlation between the number of observed pillars and the maturation stage of the glomerulus, since the pillars we found were in more mature glomeruli. On the other hand, the number of identified pillars in our research was small probably due to the strict criteria of our methodology or the type of tissue we used. Consequently, we do not have enough data to undeniably establish a correlation between pillar formation and glomerular developmental stage.

While TEM analysis is very reliable in tissues with a high density of intraluminal pillars, such as tumors [22], using it in a normally developing tissue, such as our model can be very time-consuming and laborious, without yielding enough results for quantification and statistical analysis. However, a very

promising correlative and quantitative technique has been recently developed, which could be used in the setup of the porcine metanephros in order to study IA. This microangio-CT based imaging approach for the 3D visualization of the entire vasculature down to the capillary level has been used in the murine hind limb [38] and kidney [39]. This technique will allow for the definition of sites of interest within the tissue which can then be further analyzed with optical or electron microscopy.

In conclusion, this study demonstrates the presence of intussusceptive pillars in glomerular capillaries of the porcine developing kidney. In the initial stages of the intraluminal pillar formation only endothelial cell protrusions are involved in the pillar construction. A pillar-like structure resembling a more mature pillar consists of a core of ECM covered by endothelium. The absence of perivascular cells in the formation of both structures leads to the hypothesis that these cells are involved at a later stage of pillar maturation.

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Disclosure statement

The authors have no conflicts of interest to declare.

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Figures

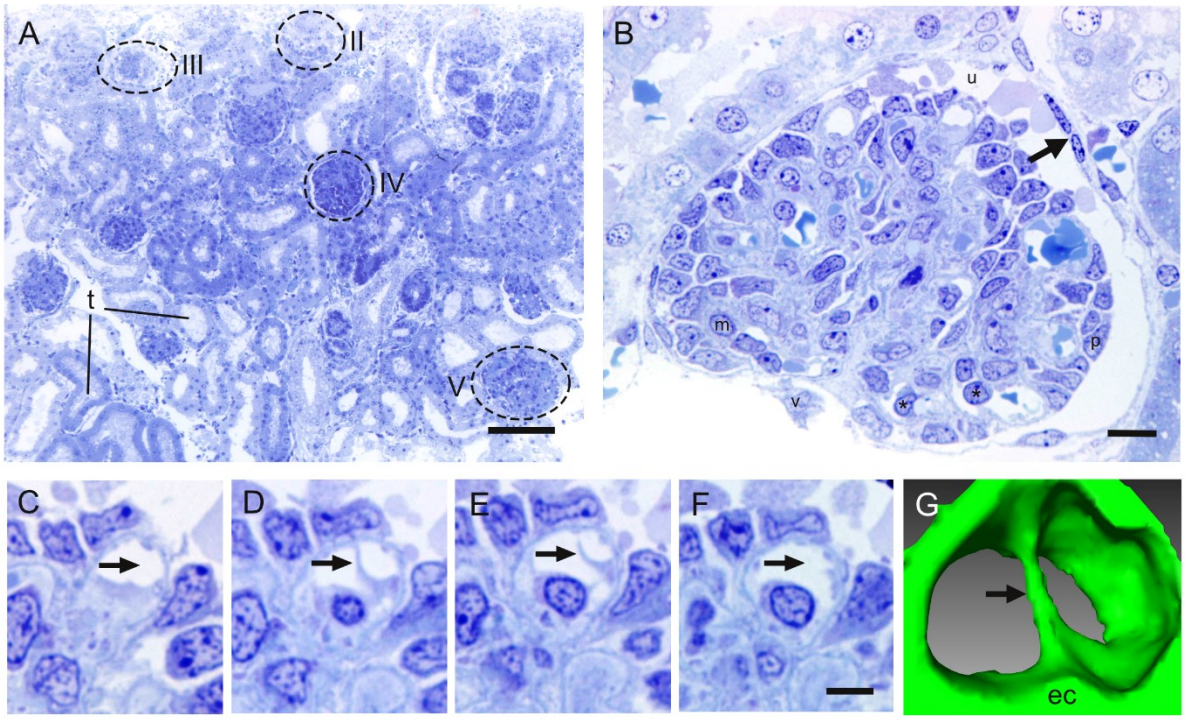

Fig. 1. Glomerular morphology of a porcine fetus (E55) and the presence of an intussusceptive pillar in the glomerular capillary. A) S-shaped bodies (II) and metanephric glomeruli of different maturation stages (III, IV and V) are located in the cortex of the metanephros. Tubuli (t) are located in the kidney medulla. Scale bar: 100 µm. B) Higher magnification of a stage IV metanephric glomerulus in which the vascular (v) and urinary poles (u) are depicted. The glomerular tuft is enclosed in Bowman's capsule (B). Endothelial cells (arrows), intraglomerular mesangial cells (*) and podocytes (p) are indicated. Scale bar: 10 µm. C-F) Consecutive semithin sections demonstrating an intraluminal tissue pillar within a glomerular capillary of the stage IV glomerulus. The open lumen of the vessel in C and F indicates the emergence and end of the pillar, respectively. The width of the pillar is 1.2 µm close to the endothelium and 0.59 µm in the middle of the lumen. Scale bar: 5 µm. G) 3D reconstruction of the intraluminal pillar represented in C-F. The endothelial cell wall (ec) and the surrounding tissue are indicated in green. The arrow points to the intraluminal pillar.

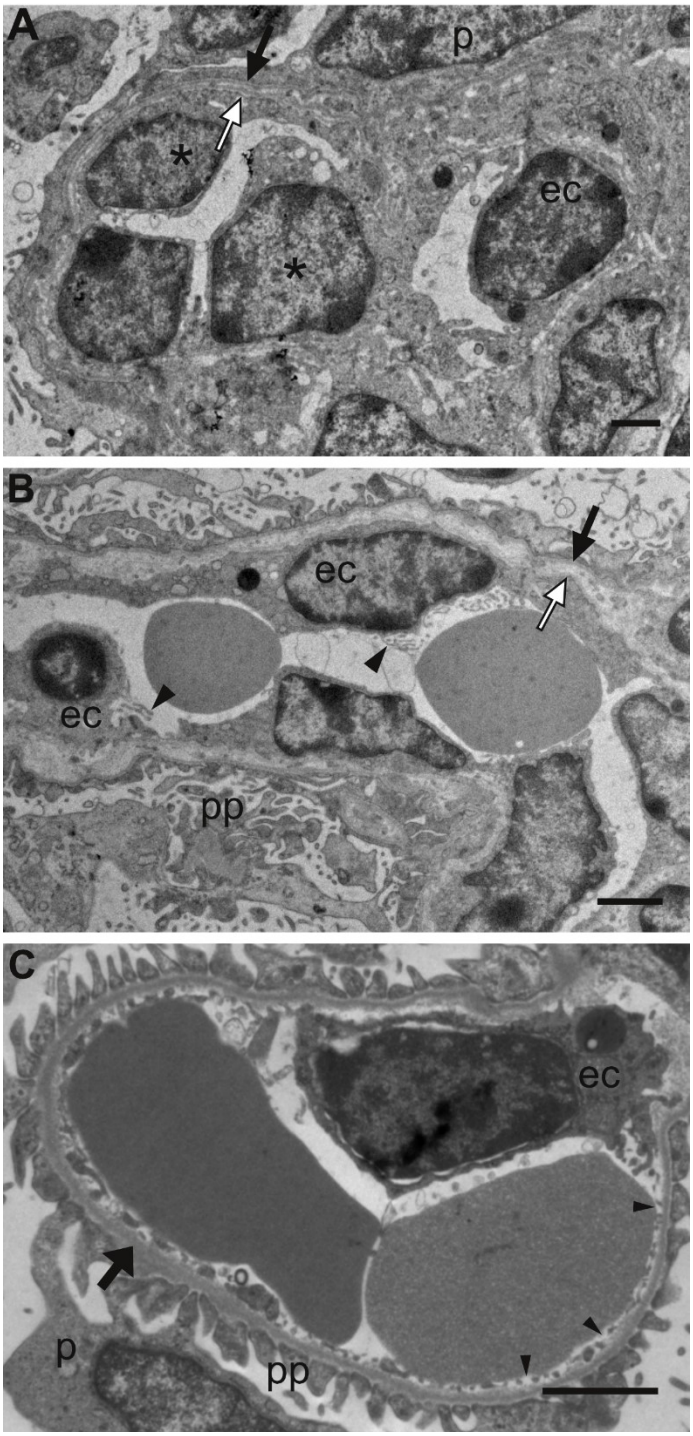

Fig. 2. Capillary phenotypes in the developing metanephric glomeruli of a porcine fetus (E64). A) Immature capillary of a stage III glomerulus showing active endothelium: thickened endothelial cells (ec), numerous organelles in the cytoplasm, nuclei with enlarged amount of euchromatin (*). The endothelium is continuous and the podocytes (p) have not developed foot processes yet. The epithelial and endothelial basal laminae are in close contact, but separated (arrows). B) Maturing capillary of a stage IV glomerulus exhibiting a few intraluminal protrusions (arrowheads). The endothelium is not fenestrated. In this stage, formation of podocyte processes (pp) is initiated. The endothelial and epithelial basal laminae are further separated (arrows). . C) Mature glomerular capillary of a stage V glomerulus displaying thin endothelial cells (ec) with sparse organelles. Endothelial fenestrations (arrowheads) are present. The mature glomerular basement membrane (arrow) is prominent and the podocyte processes are fully developed. p: podocyte. Scale bars: 2 μm.

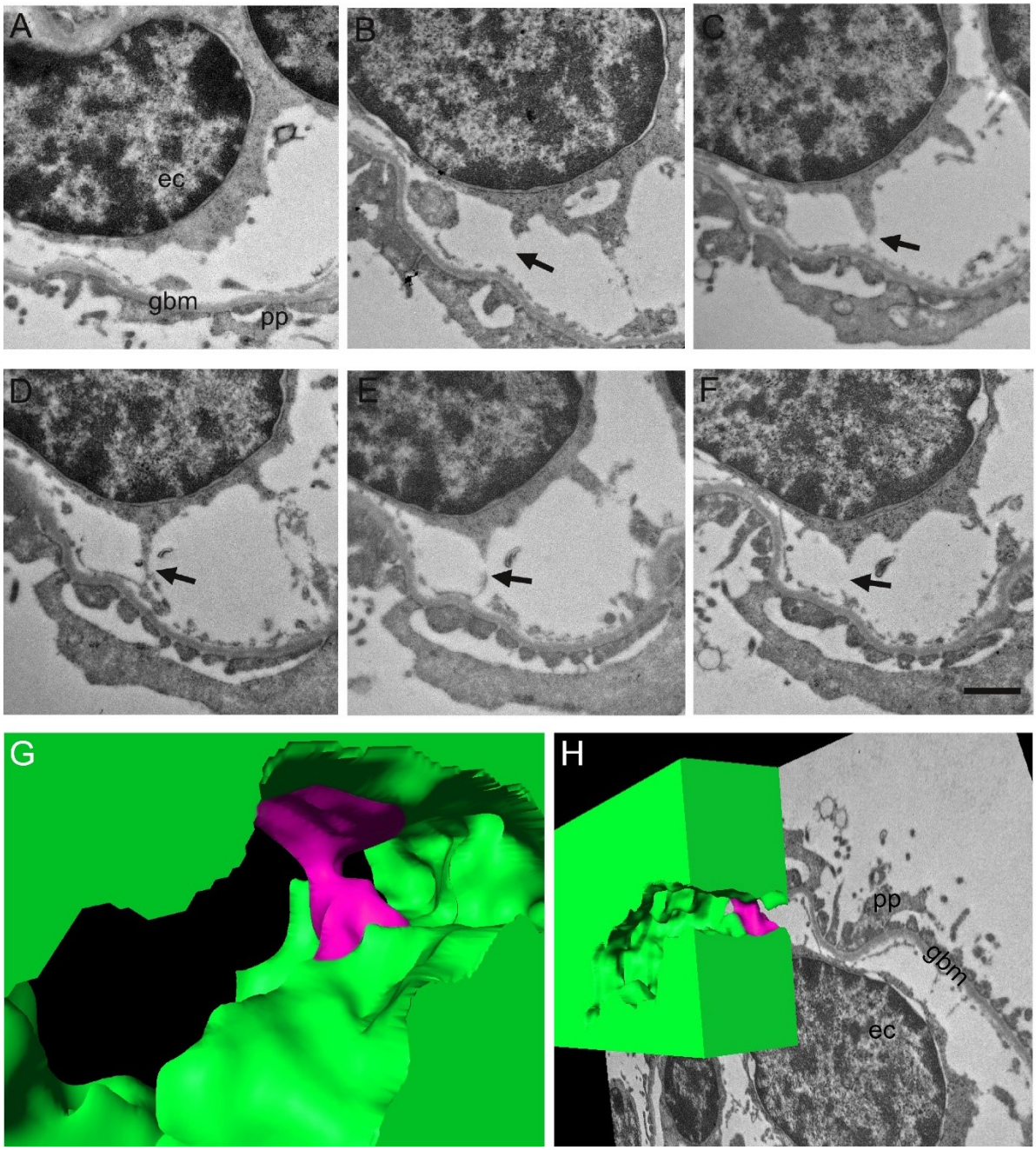

Fig. 3. Nascent intussusceptive pillar in the glomerular capillary of a porcine fetus (E64). . The capillary of the stage V glomerulus has a fenestrated endothelium and is enveloped by podocyte foot processes (pp). The nascent pillar (arrow), which consists of endothelial cytoplasm and has maximum width of 0.4µm, is present in 16 consecutive sections. A) section 24, B) section 29, C) section 33, D) section 35, E) section 37, F) section 39. Scale bar: 1 µm. G) 3D reconstruction of the intraluminal pillar represented in A-F. The endothelial cell wall (ec) and the surrounding tissue are indicated in green. Arrow points to the intraluminal pillar. H) Superimposition of the 3D reconstructed pillar (arrow) on the ultrathin section for better visualization of its position in the capillary. Glomerular basement membrane (gbm).

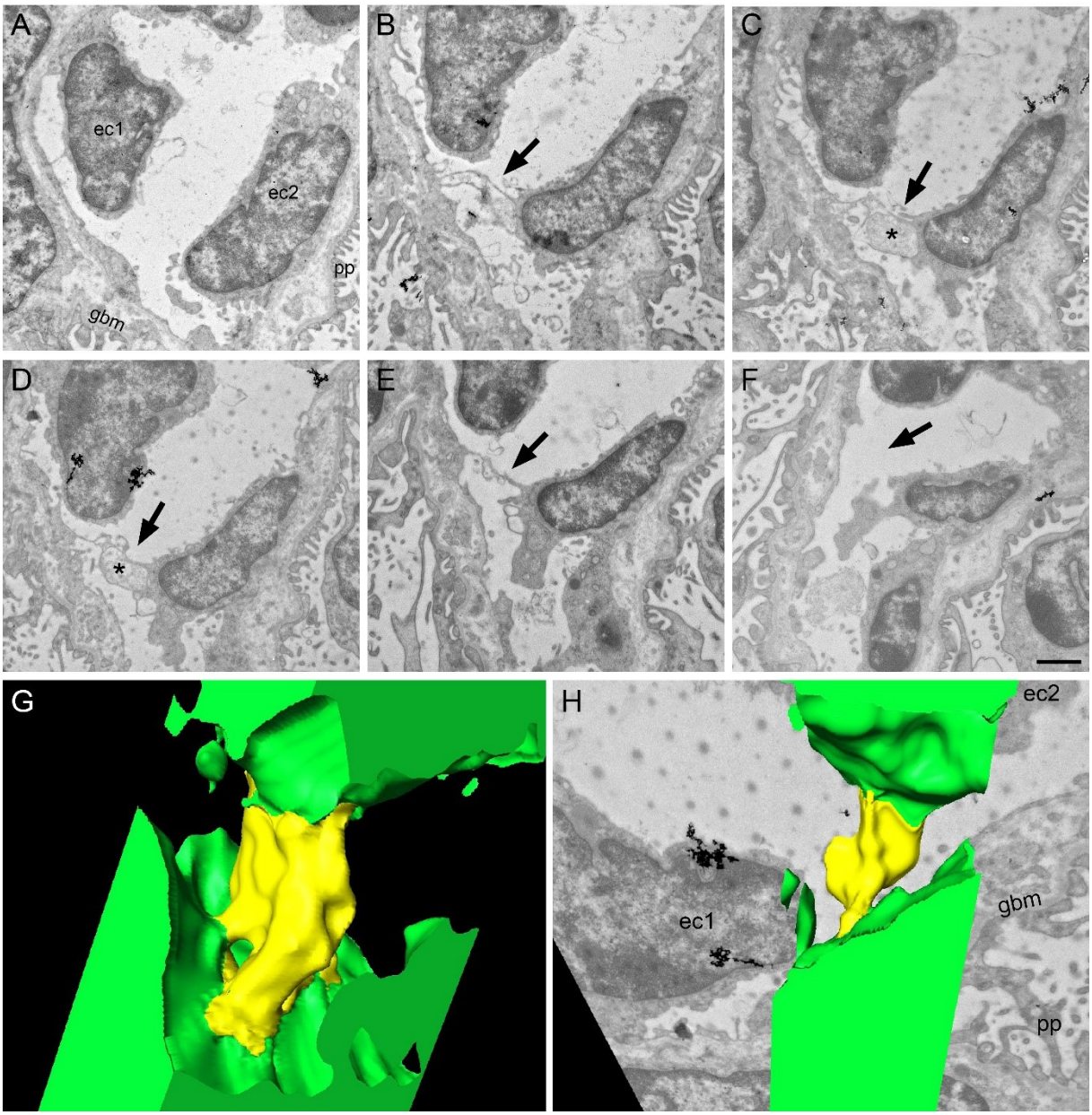

Fig. 4. Intussusceptive pillar-like structure containing ECM in the glomerular capillary of a porcine fetus (E98). The capillary of the stage IV glomerulus consists of multiple endothelial cells (ec) with a continuous endothelium. The podocytes enveloping the capillary have interdigitated foot processes (pp). The pillar-like structure (arrow) consists of extracellular matrix (*) and is covered by endothelium. The structure's maximum width is 1.2 µm and it is present in 17 consecutive sections. A) section 12, B) section 18, C) section 20, D) section 22, E) section 24, F) section 28. Scale bar: 1 µm. . G) 3D reconstruction of the pillar-like structure represented in A-F. The endothelial cells and the surrounding tissue are indicated in green whereas the endothelial extensions covering the ECM are indicated in yellow. H) Different angle of the 3D structure after the excavation of the surrounding tissue I) Superimposition of the 3D reconstructed structure (arrow) on the ultrathin section for better visualization of its position in the capillary. Glomerular basement membrane (gbm).

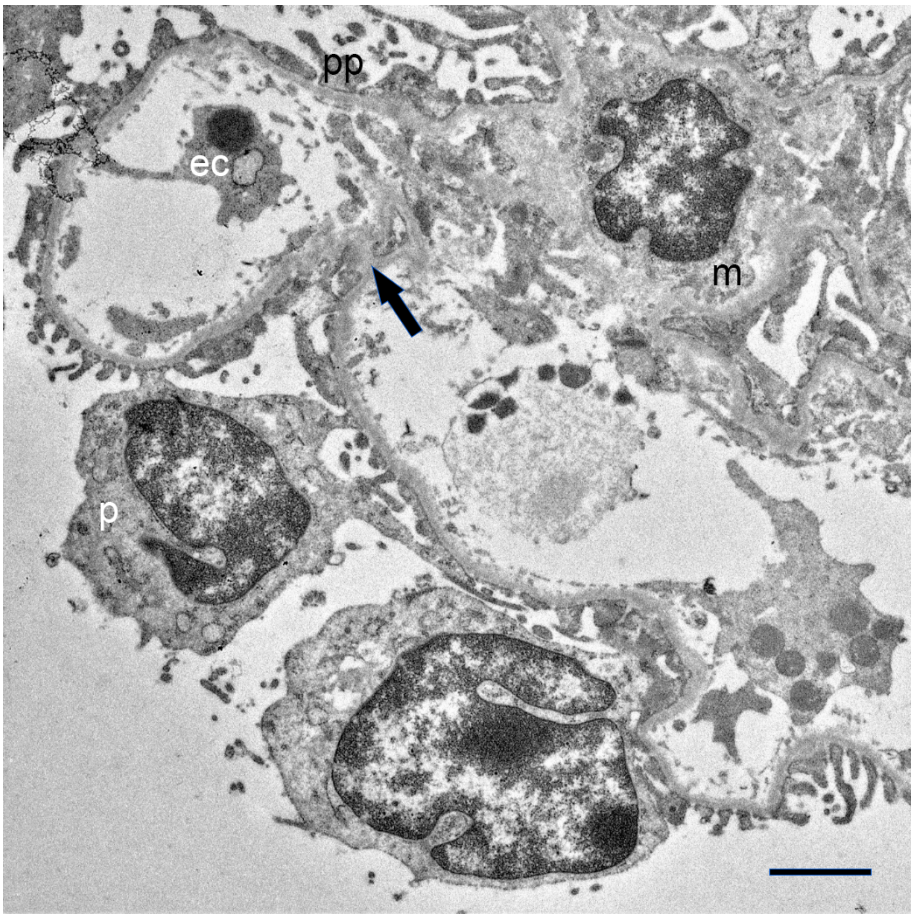

Fig. 5. Capillary bifurcation in the stage V glomerulus of a porcine fetus (E64). The splitting of the capillary begins from the endothelial wall opposite from the mesangial cell (m). Glomerular basement membrane (arrow) and podocyte processes (pp) follow the endothelial invagination. Ec: endothelial cell, p: podocyte. Scale bar: 2 μm.